TRANSGENIC MODEL OF HUMAN OXIDATIVE STRESS

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Field of the Invention

The present invention concerns transgenic animals, particularly transgenic mice, that are useful as models of human oxidative stress.

Background of the Invention

Nitric oxide is a molecule associated with human diseases of the brain and of peripheral tissues. Pathological roles for nitric oxide include cytotoxic and cytostatic effects upon bacterial invaders and toward cancer cells. The physiological roles for nitric oxide (NO) as an intra-cellular and inter-cellular messenger are the focus of intense investigation. Depending upon the situation tested, the co-localization of nitric oxide with diseased and dying cells, particularly in inflammatory conditions, suggest that NO plays an active role in protecting healthy cells from oxidative challenge or alternatively, directly participates in cellular degeneration.

Three different Nitric Oxide Synthase enzymes (NOS) convert arginine into citrulline and nitric oxide. The eNOS form associated with endothelial cells (NOS3 gene) and the nNOS form associated with neuronal cells (NOS1 gene), appear to constitutitvely produce low levels of nitric oxide. In contrast, phagocytic cells like macrophages and microglia contain an immunological form known as iNOS (also known as inducible NOS)(NOS2 gene) which can be induced to release large amounts of nitric oxide. iNOS activity is highly regulated at the level of transcription. To further complicate this picture, iNOS activity is regulated in a species specific manner

such that treatment with an inducer like lipopolysaccharide, stimulates large increases in mouse-iNOS activity as measured by nitric oxide release, with virtually no effect on human-iNOS activity (Colton et al., 1996). In addition to species specific stimulation, mice display a high-output nitric oxide release system while human macrophages, even when maximally stimulated, display a low-output nitric oxide release system (Ding et al., 1997; Weinberg et al., 1995). In comparision, the amounts of nitric oxide produced by eNOS and nNOS are low and appear to be about the same in mice and humans (Wink, 1997). Thus, the use of mice in models of human inflammatory diseases, where nitric oxide is thought to play a critical role, should be rigorously questioned on the grounds that iNOS activity differs greatly in mice and men (or women).

In view of the foregoing, there is a need for a mouse model of human iNOS function that would be useful as a model of human oxidative stress.

Summary of the Invention

Disclosed is a transgenic mouse whose germ cells and somatic cells contain *(i)* an inactive mouse inducible nitric oxide synthase gene, and *(ii)* a transgene encoding the human inducible nitric oxide synthase gene, with the transgene including all regulatory elements of the human inducible nitric oxide synthase gene necessary for human patterns of expression of said transgene in said transgenic mouse. The mice of the invention may contain one or two alleles for the human inducible nitric oxide synthase gene (i.e., one or two NOS2 alleles).

Mice of the invention are useful as models of human inflammatory disease, including but not limited to Alzheimer's disease, Multiple Sclerosis, Inflammatory Bowel Disease, and Rheumatoid Arthritis. Thus, the ability of a compound to induce such diseases may be determined or screened by administering a test compound to an animal of the invention and then monitoring the animal for the development of the disease (*e.g.* by monitoring for one or more sign, symptom or indicia of such a disease such asan underlying physiological event correlated to the disease). Further, the ability of a compound to treat such diseases may be determined or screened by administering a test compound to an animal of the invention and then monitoring that animal for treatment of the disease (*e.g.*, the alleviation, reduction, arresting or slowing of the progress of one or more sign, symptom or indicia of such a disease).

The present invention is explained in greater detail in the specification set forth below.

Detailed Description of the Invention

The human NOS2 gene is known and can be obtained in accordance with known techniques. *See, e.g.,* Spitsin, S. et al., *Mol. Med.* **2**, 226-235 (1996); Vitek, M. et al., *BBRC* **240**, 391-394 (1997).

Mice containing an inactive or inactivated mouse inducible nitric oxide synthase gene (e.g., knockout mice) are known and available from commercial sources (*e.g.*, Jackson Labs, Bar Harbor, Maine, USA).

The production of transgenic mice can be carried out in view of the disclosure provided herein and in light of techniques known to those skilled in the art, such as described in U.S. Patents Nos. 5,767,337 to Roses et al.; 5,569,827 to Kessous-Elbaz et al.; and 5,569,824 to Donehower et al. (the disclosures of which applicants specifically intend to be incorporated by reference herein in their entirety).

By "human pattern of expression" is meant that the transgene is expressed in mouse phagocytic cells, particularly activated macrophages and/or activated microglia, and more particularly is meant that lipopolysaccharide (LPS) inducers induce essentially no increase in iNOS activity in phagocytic cells of a mouse of the invention as measured by nitric oxide release as compared to the corresponding wild-type mouse, and the phagocytic cells display low-output nitric oxide release in a mouse of the invention as compared to the corresponding wild-type mouse even when maximally stimulated. In one specific example, a mouse pattern of expression in elicited peritoneal macrophages from the mouse would include stimulation of nitric oxide production by LPS or IFN-gamma, and a human pattern of expression would be a lack of nitric oxide production following stimulation with these agents.

By "corresponding wild-type mouse" is meant a mouse that does not contain the said transgene, and does contain at least one active mouse inducible nitric oxide synthase gene (e.g., one or two mouse NOS2 alleles).

To demonstrate how human iNOS contributes to human inflammatory diseases, described herein are transgenic mice lacking the mouse NOS2 gene (muNOS2 -/-) and containing at least one copy of the human NOS2 gene (huNOS +/0) which are called "Humanized-NOS2" transgenic mice. Specifically disclosed are

how to clone and characterize a PAC clone of human genomic DNA containing the entire 40 kBp of the human NOS2 gene and at least 15 kBp of its promoter-regulatory sequences, and the use of this PAC clone to create and characterize transgenic mice containing the human NOS2 gene. Transgenic mice containing the human NOS2 gene are mated to mice lacking the mouse NOS2 gene (available from Jackson Labs) to generate a hybrid transgenic mouse expressing only human iNOS (NOS2) enzyme, i.e. a "Humanized-NOS2" transgenic mouse. These mice are characterized for the expression of human NOS2 gene products.

An object of the invention is to produce a transgenic mouse whose physiological regulation of iNOS activity mimics that of humans. iNOS activity is tested in the mice to confirm it is regulated in a human-specific or in a mouse-specific pattern in these "Humanized-NOS2" transgenic mice. When the human NOS2 promoter/reporter construct is placed into mouse cells, its activity appears to mimic the human-specific pattern and not the mouse activity pattern (Mitsuda et al., 1997; Spitsin et al., 1996). As described herein, the "Humanized-NOS2" transgenic mice also display a human-specific pattern of activity. With such mice, the role of iNOS and its nitric oxide products can be better defined in terms of how humans respond to stimuli typically associated with inflammation andneurodegeneration.

To use the mice as models of human disease, mice are stressed by injecting with myelin fractions to generate Experimental Allergic Encephalomyelitis (EAE), which is a model of Multiple Sclerosis (MS) in humans, and compare the severity of disease in wild-type, NOS2-Knockout and "Humanized-NOS2" transgenic mice treated with and without various inhibitors of Nitric Oxide Synthtase. Adjuvant Induced Arthritis (AIA), which is a model of rheumatoid arthritis in humans, is also tested in these mice in a similar fashion. As the balance between superoxide and nitric oxide can define pro-oxidant and anti-oxidant conditions, a human specific pattern of nitric oxide production may correctly predict the role of nitric oxide in human pathological conditions. These "humanized-NOS2" transgenic animals are thus useful as a model to test NOS inhibitors and drugs affecting the arginine cycle.

Virtually all sucessful screening efforts to find drugs that effectively improve human disease conditions require those drugs to be tested in an animal model. A physiologically-relevant model of human nitric oxide production is herein provided. These animals serve as useful models of nitric oxide production as it pertains to the

generation of oxidative stress, which is implicated in the inflammation and destruction of cells in a wide variety of human inflammatory diseases. An important focus for such study is to measure the pro-oxidant and anti-oxidant potentials of nitric oxide in mouse models of inflammatory disease. It is well known that nitric oxide can combine with superoxide to form the strong oxidant, peroxynitrite, which, for example, can attack tyrosines in proteins to form nitrotyrosine residues as can be detected with monoclonal antibodies (Beckman, 1996). Since nitric oxide is also a radical, it can combine with the peroxynitrite radical to generate a non-radical species with the net effect of inhibiting, for example, protein and lipid oxidation. Thus, drugs which modulate nitric oxide production can be tested in the "Humanized-NOS2" animals described herein for their pro-oxidant and/or anti-oxidant potentials, their ability to relieve pain and suffering associated with inflammation and cell destruction in tissues brought about by release of inflammatory modulators.

As an additional utility, the protein products of genes associated with Alzheimer's disease (AD) can stimulate nitric oxide production. Using primed human monocyte derived macrophages as models of microglia, apolipoprotein-E can stimulate nitric oxide production. These same cells fail to produce nitric oxide in response to treatment with Amyloid-beta peptide (Abeta). Since mouse cells do produce nitric oxide in response to Abeta treatment (Meda et al., 1995), mice differ from humans in their response to stimulation of nitric oxide production (see also Walker et al., 1995). Since studies of microglial-mediated production of nitric oxide in human brains are limited by tissue availability, this "Humanized-NOS2" transgenic mouse provides a convenient animal model in which to study nitric oxide production relevant to that found in humans under physiological and pathological conditions.

While the present invention has been explained primarily with reference to mice, it will be appreciated that the invention can be implemented with other mammalian species, such as rats, dogs, cats, and monkeys, in accordance with known techniques, or techniques that will be apparent to those skilled in the relevant arts.

The present invention is explained in greater detail in the following non-limiting examples.

EXAMPLE 1

Cloning and Characterizing the Human-NOS2 gene

Several PAC clones of human genomic DNA are obtained that are positive for the Promoter/Exon-1 region and for the Exon-27 region of the human NOS2 gene. Each clone that contained both the 5' and 3' ends of the human NOS2 gene, is subjected to additional PCR reactions with primer pairs to each of the 27 Exons. Each of these Exon-specific PCR products is sequenced and compared to the reported sequences. A restriction map of each of these clones is made and compared to the reported restriction enzyme map to also ensure that the PAC clone is correct. Additional mapping employs Southern blots of the positive PAC clones and of human genomic DNA, digested with Hind III, which will be hybridized with Exon-specific DNA probes (Vitek et al., 1984; Xu et al., 1994). The PAC clone(s) containing all of the human Exons, with the correct DNA sequences and restriction maps matching human genomic DNA, are then used for generating the "human-NOS2" transgenic mouse.

EXAMPLE 2

Making the "human-NOS2" Transgenic Mouse

The entire human-NOS2 gene is used to generate the "human-NOS2" transgenic mouse by conventional methods with a service provider such as DNX (Princeton, New Jersey, USA). Briefly, DNA from a PAC clone that meets all of the criteria listed above for containing a human NOS2 gene, is used to generate transgenic mice. PAC clone #2 contains both the Promoter/Exon-1 region and the Exon-27 region of the human--NOS2 gene in about 100 kBp of human genomic DNA. PAC clone DNA is purified according to the instructions provided by the provider. PAC clone #2 is linearized with Not I. Not I is reported not to cleave the human NOS2 gene and it does not cleave the human NOS2 gene in PAC clone #2 which has been isolated, but Not I does cleave at both ends of the PAC vector as confirmed by pulse-field agarose gel electrophoresis. Linearized DNA is then injected into the pronucleus of a fertilized mouse egg by the service provider. The injected eggs are reimplanted into pseudopregnant female mice who then give birth to the babies, the putative F_0 founders.

Genomic DNA extracted from snips of the end of the tails (snips of typically 2 to 5 mm in length) of the fully weaned pups are used to obtain the genotypes of each animal including putative "human-NOS2" transgenic mice (Xu et al. 1996a). After about 3 weeks when the pups are weaned, tail snips are taken and identification tags placed on each animal's ears. Genomic DNA is then extracted from the tails of these F_0 mice (putative founders). Tail snips are minced with a fresh razor blade, placed in 0.4 ml of TE (10mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 7.4), extracted twice with 2 volumes of phenol:chloroform:isoamyl alcohol (50:48:2), re-extracted with 2 volumes of chloroform:isoamyl alcohol (24:1) and re-extracted with 2 volumes of ether. Residual ether in the aqueous phase is removed by drying under a stream of nitrogen gas. This DNA is genotyped by PCR for the presence of the human NOS2 gene using human-specific DNA probes from human NOS2's Promoter/Exon-1 region (Forward Promoter Primer = CCTTTCCCTTCCAAAAACCTC; Reverse Exon-1 Primer = TCACCCAACCCACCTCTTTC to give a 345 bp product). A second PCR from the mouse TAU gene is also performed as a control for the PCR technique and for the presence of mouse DNA using a forward primer (TAU Exon-14 Forward Primer = TTGGCACTTCGATGATGACCTC) and reverse primer (TAU Exon-14 Reverse Primer = CATTGTGACGTGTGATGAGGGG) which give a PCR product of 420 bp whose sequence matches that reported (Andreadis et al., 1992). Southern blots of genomic DNA digested with Hind III and hybridized with the pIN-2 probe (spanning positions +2401 to +4203 of the human NOS2 cDNA which corresponds to Exon-18 to Exon-27 of the human NOS2 gene), are also employed. In this Southern paradigm, human NOS2 gene gives multiple bands of 12, 9.5, 8, 6, 4.5, 4 and 3.6 kBp, while mouse genomic DNA gives only one band of 7.6 kBp (Xu et al. 1994). Hybrid mice containing the human NOS2 and the mouse NOS2 genes contain the mouse-specific 7.6 kBp band and all 7 of the human-specific bands. In an alternative Southern paradigm, BamHI digest of genomic DNA probed with oligonucleotides from the sequence for Exon-18 are predicted to give a 14 kBp band from wild-type mouse DNA, a 10 kBp band from mu-NOS2-knockout DNA and a 2 kBp band from human DNA (Laubach et al., 1995; Xu et al. 1996b). Non-transgenics have a 14 kBp band hybridizing to an Exon-18 probe, while the hemizygous "human NOS2" transgenic mouse display bands at 14 kBp and at 2 kBp hybridizing to the Exon-18 probe.

F_0 mice containing the human NOS2 gene are mated to wild-type mice and their offspring (F_1 generation), ear tagged and genotyped for the presence of the human-NOS2 gene from tail snip DNAs as described above. F_1 mice carrying the human NOS2 gene have passed this gene through the germ line and are hemizygous. These mice are then be used for matings as described below.

EXAMPLE 3

Breeding and Identifying a "Humanized-NOS2" Transgenic Mouse

Mice hemizygous for the human NOS2 gene (generated as described above) are mated to NOS2 knockout mice to generate a transgenic mouse expressing only human NOS2 gene products. The simplest way to generate such a mouse is to mate the hemizygous human NOS2 mice (which are +/0 with respect to the human NOS2 gene and +/+ with respect to the murine NOS2 gene) with homozygous NOS2 knockout mice (which are -/- with respect to the murine NOS2 gene and lack functional muNOS2 genes). The resulting F1 hybrids of this first cross display an "H-Mm" genotype (arbitrary letter designation) which is +/0 for the huNOS2 gene and +/- for the muNOS2 gene and a "Mm" genotype which is -/- for huNOS2 and +/- for muNOS2. Equal numbers of the "H-Mm" and "Mm" genotypes are observed in this cross. Mice of the "H-Mm" genotype are then mated again to the muNOS2 knockout mice (homozygous -/- for functional muNOS2) to generate F2 hybrid mice that display a "H-mm" genotype which is +/0 for huNOS2 gene and -/- for muNOS2 gene and a "Mm" genotype which is -/- for huNOS2 and +/- for muNOS2. Equal numbers of the "H-mm" and the "Mm" genotypes are observed in this cross. The "H-mm" genotype mice are then characterized for the expression of only human NOS2 gene products and the nitric oxide produced from the human NOS2 gene products is found to be expressed in a human specific pattern.

Several methods are available to confirm the genotype of the transgenic mice. A PCR strategy with a promoter/Exon-1 primer pair gives a specific band from the human NOS2 transgene DNA of about 477 bp. Also, as an alternative Southern paradigm, BamH1 digests of genomic DNA probed with oligonucleotides from the sequence of Exon-18 are predicted to give a 14 kBp band from wild-type mouse DNA, a 10 kBp band from muNOS2 knockout DNA and a 2 kBp band from human DNA (Laubach et al, 1995; Xu et al 1996b). "H-Mm" mice display Exon-18

hybridizing bands at 2 kBp (from huNOS2), 14 kBp (from muNOS2) and 10 kBp (from the disrupted muNOS2 gene of knockout mice). "Mm" mice display Exon-18 hybridizing bands at 14 kBp and at 10 kBp. "H-mm" mice display Exon-18 hybridizing bands at 2 kBp and at 10 kBp. In this way, one can use Southern blots of tail snip DNA to diagnose the genotype of each of the transgenic hybrid mouse strains. As another alternative, one can perform test matings by crossing "H-mm" mice to "mm" muNOS2 knockout mice which will give half of the offspring carrying the human NOS2 transgene and the other half lacking it.

EXAMPLE 4

Characterizing Human-NOS2 Gene Products

Human NOS2 transgene expression is assessed at the RNA and at the protein levels. To measure human NOS2-mRNA, RNA is extracted from brain, denatured and Northern-blot the RNA, hybridize to the human-specific NOS2 probe and examined for bands of about 4.2 kB in size as reported by Charles et al. (1993). Although mouse NOS2 mRNA has been reported to also run in the 4 kB range (Laubach et al. 1995), it should not cross-hybridize to our probe which derives from human NOS2 Exons-1, -2 and -3 which are specific for the human-NOS2 sequence and do not share homology with the mouse NOS2 gene or with any non-human-NOS2 sequence reported in Genbank. As a positive control for hybridization, probe with an APP cDNA for bands in the 3 kB range (Vitek et al. 1988). If the human-NOS2 transgene is being transcribed and post-transcritionally spliced in mice, in a manner similar to that found in humans, then animals carrying the human-NOS2 transgene express RNA transcripts that hybridize to the human-specific NOS2 probe while wild-type mice, lacking the transgene, will not display hybridizing transcripts as visualized on Northern blots.

The human iNOS protein and the mouse iNOS protein share high homology at the amino acid sequence level. Although several commercially available antibodies recognize 130 kDa bands on Western blots, the inability of these antibodies to discriminate human from mouse proteins leads us to propose this method only if Northern blots fail to provide the desired information. Thus, a combination of DNA-genotype and bands on a Western could be used to diagnose the presence of human iNOS protein product of the human NOS2 transgene.

EXAMPLE 5

Uses of Humanized-NOS2 Mice

A "Humanized-NOS2" transgenic mouse expressing only human iNOS protein, is initially useful to test whether the regulation of human-NOS2 induction follows a human-specific or a mouse-specific pattern when in a mouse host. The induction pattern is measured following stimulation of nitric oxide synthase activity with various inducers by indirectly measuring nitrite as a marker of enzymatically-generated nitric oxide release. Since the levels of enzymatic activity of eNOS and nNOS are typically constitutive in peritoneal macrophages and represent a fraction of the inducible iNOS activity which they possess, then peritoneal macrophages are harvested from wild-type, muNOS2 knockout and from "Humanized-NOS2" transgenic mice for placement in culture and treatment with stimulating agents followed by nitrite (nitric oxide) measurement. Laubach et al. (1995) report that elicited peritoneal macrophages from wild-type mice, treated with lipopolysaccharide (LPS) and Interferon-gamma (IFN-gamma), have about 15 times more NOS activity than similarly treated cells of NOS2 knockout mice. MacMicking et al. report that nitrite levels in elicited peritoneal macrophages from wild-type mice treated with LPS and/or IFN-gamma, are at least 15 fold higher than similarly treated cells of a different NOS2 knockout mouse (MacMicking et al., 1995). Weinberg et al. (1995) report that human peritoneal macrophages produce little or no nitrite (ie. nitric oxide) before or after treatment with LPS, IFN-gamma or both. These data indicate that a mouse-specific pattern of nitric oxide production in elicited peritoneal macrophages would include stimulation by LPS or IFN-gamma and that a human-specific pattern would be a lack of nitric oxide production following stimulation with these agents. As suggested by Ding et al (1997), iNOS activity and nitrite release are increased following stimulation of human microglia, the brain-specific macrophage, with a combination of Interferon-gamma and Interleukin-1 beta (IL-1beta). This combination of IFN-gamma and IL-1beta are used as a positive control to show that the peritoneal macrophages from various mice are capable of showing an inducible rise in nitrite levels. Alternatively, one can culture microglia from the various mice and test their responses to these inducers.

Specific methods for obtaining peritoneal macrophages, measuring nitrite via Greiss reaction and culturing mouse brain microglia are found in Laubach et al. and

Colton et al. (Colton and Gilbert, 1993; Laubach et al., 1995). Briefly, macrophages are elicited by intraperitoneal (i.p.) injection of 2 ml of 4% Brewer thioglycollate (Difco). Four days following injection, cells are harvested by washing out the peritoneal cavity with 20 ml of cold phosphate buffered saline and the entire solution centrifuged to pellet the cells. Cells are resuspended in 20 ml of RPMI 1640 with 10% fetal bovine serum (RPMI-FBS) (Gibco-BRL-Life Technologies), centrifuged, cell pellets resuspended in RPMI-FBS and plated in 4 x 35 mm tissue culture dishes per mouse. After 3 hours at 37°C to allow cells to attach, non-adherent cells are removed with 3 washes of RPMI-FBS and adherent cells used for stimulation experiments. Adherent cells are treated with nothing (as a non-stimulated control), murine IFN-gamma (100 units/ml, Boehringer Mannheim) or LPS (1 µg/ml, E. coli 055:B5, Sigma) for 12, 24, 36, and 48 hours. At each time point, quadruplicate 50 µl aliquots of conditioned media are removed to 96 well tissue culture plates and nitrite levels assayed with the Greiss reaction. Briefly, an equal 50 µl aliquot of Greiss reagent (a 1:1 solution of 0.1% N-(1-napthyl)ethylenediamine dihydrochloride: 1% sulfanilamide in 5% phosphoric acid), incubated for 10 minutes at room temperature, and absorbance read at 544 nm of a plate reader. Sodium nitrite standards were employed to generate a standard curve of the colorimetric response. The method of Colton et al. is employed to prepare primary cultures of mouse brain microglia and test their responses to stimuli with the Greiss reaction listed above (Colton and Gilbert, 1993).

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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